IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant	: Charlotte Albaek Thrue et al.	Art Unit	: 1635
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Title	: ANTISENSE DESIGN		

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Declaration of Miriam Frieden

Miriam Frieden hereby declares as follows:

1. I am an inventor of the above-captioned patent application. I am Director Business Development, Santaris Pharma A/S (Hørsholm, Denmark), owner of this patent application. I have a Ph.D. in Chemistry from the Universitat de Barcelona, Spain (2000), a Master of Science in Chemistry from King's College, University of London, UK (2006), and a Degree in Chemistry from Universitat de Barcelona, Spain (1995). My curriculum vitae is attached as Exhibit A. A list of my publications is attached as Exhibit B.

2. The effect of the presence of a deoxynucleotides (DNA) at the 3' end of an oligonucleotide rather than a beta-D-oxy –LNA (LNA) was examined using two pairs of oligonucleotides. As detailed below, all four of the oligonucleotides in the two pairs had the same sequence and length are were targeted to luciferase. The four oligonucleotides were tested for their ability to reduce luciferase expression in HeLa cells using the experimental protocol described below in paragraph 6.

3. One pair of oligonucleotides (Oligo A and Oligo B), depicted below, was identical in sequence, length and internucleotide linkages. They differed in that one member (Oligo A) had a

LNA at the 3' terminus and in the other member (Oligo B) had a non-locked nucleotide (a DNA) at the 3' terminus.

		Luciferase expression 50 nM oligo	2 nM
Control Oligo	$t_st_sc_sc_sg_st_sc_sa_st_sc_sg_st_sc_st_st_st$	29.8±4.7	65.8±8.1
Oligo A	$T_sT_sC_sc_sg_st_sc_sa_st_sc_sg_st_sC_xT_sT_sT$	0.7±0.2	3.0±1.0
Oligo B	$T_sT_sC_sc_sg_st_sc_sa_st_sc_sg_st_sC_sT_sT_st$	1.8±0.1	2.2±0.4

Capital Letters = oxyLNA; Lowercase Letters = DNA; s = phosphothioate bond, otherwise phosodiester bond

When these oligonucleotides were tested for their ability to reduce expression of a target gene, luficerase, it was found in the oligonucleotide (Oligo B) having a DNA at its 3' end was about as potent than the otherwise identical oligonucleotide (Oligo A) having a LNA at its 3' end. As LNA are known to bind to RNA with higher affinity than does DNA, one would expect that reduction of the LNA load by 14% would affect the potency of OligoB.

4. The other pair of oligonucleotides (Oligo C and Oligo D), depicted below, was identical in sequence and length (and identical in sequence and length to Oligos A and B above). However, one member (Oligo C) had a LNA at the 3' terminus and in the other member (Oligo D) had a non-locked nucleotide (a DNA) at the 3' terminus.

		Luciferase expression 50 nM oligo	2 nM oligo
Oligo C	$TTCc_sg_st_sc_sa_st_sc_sg_st_sCTTT$	7.1±0.4	10.7±1.5
Oligo D	$Cc_sg_st_sc_sa_st_sc_sg_st_sCTT_st$	0.2±0.1	1.1±0.4

Capital Letters = oxyLNA; Lowercase Letters = DNA; s = phosphothioate bond, otherwise phosphodiester bond

In this pair, where there are fewer phosphothioate bonds, the oligonucleotide with a DNA at its 3' end (Oligo D) was more potent than the oligonucleotide having an LNA at it 3'. This is a surprising result given the greater affinity of LNA than DNA for complementary RNA. Importantly, the oligonucleotide (Oligo D) with the DNA at the 3' end was more potent than the oligonucleotide having a LNA at the 3' end (Olgio C). In fact, by comparing Oligo D and Oligo

A, above, it can be seen that Oligo A which has a DNA at the 3' end and 10 phosphothioate bonds is more potent than Oligo A having a DNA at the 3' end and 15 phosphothioate bonds. This is a very important advantage of the oligonucleotide having a DNA at the 3' end because phosphothioate bonds are understood to confer a degree of toxicity and it is considered desirable to reduce the number of phosphothioate bonds.

5. To summarize, the experiments described in paragraph 2 and 3 demonstrate that by placing a DNA rather than a LNA at the 3' end of a gapmer oligonucleotide it is surprisingly possible to create an oligonucleotide that is more potent than an oligonucletide of the same length and sequence, but containing more phosphothioate bonds.

6. The experiments described in paragraphs 2 and 3 used the X1/5 Hela cell line (ECACC Ref. No: 95051229), which is stably transfected with a "tet-off" luciferase system. In the absence of tetracycline the luciferase gene is expressed constitutively. The expression can be measured as light in a luminometer, when the luciferase subatrate, luciferin has been added. The X1/5 Hela cell line was grown in Minimun Essential Medium Eagle (Sigma M2279) supplemented with 1x Non Essential Amino Acid (Sigma M7145), 1x Glutamax I (Invitrogen 35050-038), 10 % FBS calf serum, 25 µg/ml Gentamicin (Sigma G1397), 500 µg/ml G418 (Invitrogen 10131-027) and 300 µg/ml Hygromycin B (invitrogen 10687-010). The X1/5 Hela cells were seeded at a density of 8000 cells per well in a white 96 well plate (Nunc 136101) the day before the transfection. Before the transfection, the cells were washed one time with OptiMEM (Invitrogen) followed by addition of 40 µl of OptiMEM with 2µg/ml of Lipofectamine2000 (Invitrogen). The cells were incubated for 7 minutes before addition of the oligos. 10 µl of oligo solutions were added and the cells were incubated for 4 hours at 37°C and 5 % CO_2. After the 4 hours of incubation the cells were washed once in OptiMEM and growth medium was added (100 µl). The luciferase expression was measure the next day. Luciferase expression was measured with the Steady-Glo luciferase assay system from Promega. 100 µl of the Steady-Glo reagent was added to each well and the plate was shaken for 30s at 700rpm. The plate was read in Luminoskan Ascent instrument from ThermoLabsystems after 8minof

incubation to complete total lysis of the cells. The luciferase expression is measured as Relative Light Units per seconds (RLU/s). The data was processed in the Ascent software (v2.6) and graphs were drawn in SigmaPlot2001.

7. All statements made herein of my own knowledge are true and all statements made on information and belief are believed to be true; and further, that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the instant patent application or any patent issued thereon.

Date: 14/4/2009

Miriam Frieden, Ph.D.